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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/057258 A2

(54) Title: QUANTITATIVE RANKING OF TRANSIENT LIGAND BINDING TO TARGET BIOMOLECULES BY USE OF NUCLEAR MAGNETIC RESONANCE

(57) Abstract: The present invention relates to a new use of NMR for quantitatively ranking transient ligand binding to target biomolecules. The present invention also relates to a new method to identify ligand site obeying two-state and more complex binding behavior in a transient complex of a ligand with a target molecule, still with the use of NMR. There is also provided an efficient method to quantitate fast dissociation rates of ligands containing at least one magnetic nuclei by performing NMR relaxation dispersion experiments at different protein concentrations, enabling the evaluation of populations and exchange rates, and extending the practical applicability of the NMR relaxation dispersion experiments.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/CA 03/00014	International filing date *(day/month/year)* 10.01.2003	Priority date *(day/month/year)* 11.01.2002
International Patent Classification (IPC) or both national classification and IPC G01R33/46, G01R33/46		
Applicant NATIONAL RESEARCH COUNCIL OF CANADA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
06.08.2003	29.03.2004
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized Officer Telephone No. +49 89 2399-

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA 03/00014

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-51 as originally filed

Sequence listings part of the description, Pages

55-60 as originally filed

Claims, Numbers

1-16 received on 09.02.2004 with letter of 04.02.2004

Drawings, Sheets

1/20-20/20 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).

   ☐ the language of publication of the international application (under Rule 48.3(b)).

   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority in written form.

   ☐ furnished subsequently to this Authority in computer readable form.

   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:

   ☐ the claims, Nos.:

   ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-16
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations

see separate sheet

1. Cited documents

Reference is made to the following documents:
D1: Millet O. et al, J. Am. Chem. Soc. 2000, 122, 2867-2877;
D2: Tollinger et al, J. Am. Chem. Soc. 2001, 123, 11341-1135;
D3: US-A-5 891 643.

2. Re item V: Reasoned statement under Art. 35(2) PCT:

The invention concerns a method to identify a ligand site obeying a two-state or more complex binding behaviour in a transient complex of a ligand with a target molecule.

Document D3 may be regarded as representing the closest prior art because it discloses a method to determine the dissociation or binding affinity of various ligands to various target molecules. The method of D3 comprises
preparing a ligand with at least one nucleus detectable by NMR,
titrating a target molecule with various concentrations of the ligand, generating two-dimensional correlation spectra at each concentration of ligand and determining the dissociation constant between target molecule and ligand on the basis of differences in the spectra.

It can be considered to be a problem of the determination of dissociation rate constants according to the method of D3 that it necessitates an accurate knowledge of the concentrations of ligand and target molecules.

According to the invention, this problem is overcome by (item b of claim 1) collecting NMR relaxation dispersion profiles for the ligand nucleus at two or more magnetic fields, determining (item c of claim 1) apparent transverse relaxation rates from said dispersion profiles, collecting (item f of claim 1) NMR relaxation dispersion profiles for the ligand contacted with at least one concentration of the target molecule for every concentration of the target molecule at two or more magnetic fields, fitting (item g of claim 1) said dispersion profiles by including the predetermined relaxation rates for the free ligand and using a two-state exchange model independently for every nucleus, and independently or simultaneously for every concentration of the target molecule; and determining (item h of claim 1) a ligand site obeying a two-state binding behaviour on the basis of the fitting procedure (item g).

Although steps d)-f), if taken alone, would be known from prior art, see, for instance,

documents D1 and D2 mentioned in the description, the methods disclosed therein had not been applied to the identification of ligand sites with the steps of preparing a ligand and collecting NMRD profiles for the free ligand at two or more magnetic fields, see items a) and b) of claim 1. Consequently, the step of including relaxation rates determined with steps a) and b) of claim 1 in the fitting procedure defined in item g) would not be obvious w.r.t. documents D1 and D2.

Thus, claim 1 is inventive. The same applies to the other claims because they concern preferred embodiments or a use of the method, respectively.

WHAT IS CLAIMED IS:

1. A method to identify a ligand site obeying a two-state or more complex binding behavior in a transient complex of a ligand with a target molecule, said method comprising the steps of:

   a) preparing a ligand with at least one nucleus detectable by NMR;

   b) collecting NMR relaxation dispersion profiles for free ligand at two or more magnetic fields;

   c) determining apparent transverse relaxation rates for the nuclei detectable by NMR at two or more magnetic fields;

   d) assigning resonance peaks to the specific NMR detectable nuclei of the ligand with one- and/or multi-dimensional NMR;

   e) contacting the ligand with at least one concentration of a target molecule;

   f) collecting NMR relaxation dispersion profiles for the ligand with every concentration of the target molecule at two or more magnetic fields;

   g) fitting the NMR relaxation dispersion profiles by a two-state exchange model independently for every nucleus, and independently or simultaneously for every concentration of the target molecule; and

   h) determining a ligand site obeying a two-state binding behavior based on feasibility of extracted R_{2b} and p_b parameters or the quality of the fitting of step g).

2. The method of claim 1, wherein the ligand of step a) has at least two detectable nuclei.

3. The method of claim 1 or 2, wherein the ligand in step e) is contacted with at least two concentrations of a target molecule.

4. The method of claim 3, wherein the ligand is contacted with three concentrations of target molecules.

5. A method to determine quantitatively the dissociation rate constant (k_{off}) for a transient complex of a ligand with the target molecule comprising the steps of:

   a) Identifying a ligand site obeying a two-state or more complex binding behavior in a transient complex of a ligand with a target molecule with the method as defined in claim 1, 2, 3 or 4; and

   b) Extracting k_{off} values for the ligand sites obeying two-site or more complex exchange mechanism, said k_{off} values being a measure of the affinity of a transient complex of the ligand with the target molecule.

6. A method according to claim 1, 2, 3, 4, or 5, wherein the ligand is a polypeptide.

7. A method according to claim 1, 2, 3, 4, 5 or 6, wherein the ligand is a ^{15}N-enriched polypeptide.

8. A method according to claim 1, 2, 3, 4, 5, 6 or 7, wherein the ligand is a mixture of polypeptides and/or molecules.

9. A method according to claim 1, 2, 3, 4, 5, 6, 7 or 8, wherein the target molecule is a protein or a protein assembly.

10. Use of the method as defined in claim 1, 2, 3, 4, 5, 6, 7, 8 or 9 to determine amino acid residues with detectable NMR relaxation dispersion as a constituting binding hot-spot.

11. Use of the method as defined in claim 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 to identify two or more ligands that can be linked together to create high-affinity molecules.

12. Use of the method of claim 1, 2, 3, 4, 5, 6 7, 8 or 9 to study high-affinity protein-protein interactions or slow-dissociating ligand-target complexes.

13. The use of claim 10, 11 or 12, wherein the ligand is a polypeptide or a protein.

14. The use of claim 10, 11, 12 or 13, wherein the ligand is ^{15}N-enriched.

15. The method of any one of claims 1 to 9, wherein in step b) said NMR relaxation dispersion profiles is CPMG.

16. The method of any one of claims 1 to 9, wherein in step f) said NMR relaxation dispersion profiles is CPMG.

17. The method of any one of claims 1 to 9, where the NMR relaxation dispersion profiles are collected by a CPMG method.

18. The use of claims 10 to 14 where the NMR relaxation dispersion profiles are collected by a CPMG method.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 03/00014

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01R33/46 G01N24/08 A61K49/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K G01R G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
INSPEC, BIOSIS, EMBASE, EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 891 643 A (OLEJNICZAK EDWARD T ET AL) 6 April 1999 (1999-04-06) column 9, line 65 -column 10, line 56 ---	1-18
A	MILLET O ET AL: "The static magnetic field dependence of chemical exchange linebroadening defines the NMR chemical shift time scale" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY 29 MAR 2000 UNITED STATES, vol. 122, no. 12, 29 March 2000 (2000-03-29), pages 2867-2877, XP002245491 ISSN: 0002-7863 cited in the application * the whole document * --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2003	10/07/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Skalla, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DAVIS D G ET AL: "Direct measurements of the dissociation-rate constant for inhibitor-enzyme complexes via the T/sub 1 rho / and T/sub 2/ (CPMG) methods" JOURNAL OF MAGNETIC RESONANCE, SERIES B, JULY 1994, USA, vol. 104, no. 3, pages 266-275, XP002245492 ISSN: 1064-1866 cited in the application * section "T2 (CPMG) Experiments *	1-18
A	DUBOIS B W ET AL: "F-NMR spin-spin relaxation (T2) method for characterizing volatile anesthetic binding to proteins. Analysis of isoflurane binding to serum albumin" BIOCHEMISTRY 1992 UNITED STATES, vol. 31, no. 31, 1992, pages 7069-7076, XP002245521 ISSN: 0006-2960 cited in the application page 7071, right-hand column, paragraph 4 - paragraph 5 section "Discussion"	1-18
A	TOLLINGER M ET AL: "Slow dynamics in folded and unfolded states of an SH3 domain" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY 21 NOV 2001 UNITED STATES, vol. 123, no. 46, 21 November 2001 (2001-11-21), pages 11341-11352, XP002245522 ISSN: 0002-7863 cited in the application figures 2,9	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 03/00014

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US 5891643 A	06-04-1999	AT	201768 T	15-06-2001
		AU	711092 B2	07-10-1999
		AU	7680496 A	05-06-1997
		DE	69613146 D1	05-07-2001
		DE	69613146 T2	16-01-2003
		DK	870197 T3	27-08-2001
		EP	0870197 A2	14-10-1998
		ES	2159056 T3	16-09-2001
		GR	3036454 T3	30-11-2001
		JP	3300366 B2	08-07-2002
		JP	2002510384 T	02-04-2002
		PT	870197 T	30-11-2001
		WO	9718469 A2	22-05-1997
		US	2001004528 A1	21-06-2001
		US	5989827 A	23-11-1999
		US	2002037529 A1	28-03-2002